# High Frequency of *p16*$^{INK4A}$ Promoter Methylation in *NRAS*-Mutated Cutaneous Melanoma

Anders Jonsson[1,2], Rainer Tuominen[1,2], Eva Grafström[1], Johan Hansson[1] and Suzanne Egyhazi[1]

The *p16*$^{INK4A}$ tumor suppressor is often deleted, or otherwise inactivated, in malignant melanoma. To investigate the loss of *p16*$^{INK4A}$ in greater detail, we analyzed 77 cutaneous melanoma metastases. Of these 56 retained at least one *p16*$^{INK4A}$ allele, and 21 had biallelic deletions. Using methylation-specific PCR, direct sequencing, and immunohistochemical methods, we analyzed *p16*$^{INK4A}$ promoter methylation, mutations, and protein expression, respectively. In addition, 14 corresponding primary tumors were analyzed for protein expression. Results were compared to clinicopathological parameters and previously obtained data regarding mutations in proto-oncogenes *NRAS* and *BRAF*. Results revealed that *p16*$^{INK4A}$ promoter methylation was present in 15 of 59 (25%) metastases; nonsynonymous mutations in 9 of 56 (16%) metastases; and protein expression in 12 of 67 (18%) metastases. Protein expression was lost during progression from primary to metastatic tumors, 71% (10 of 14) and 43% (6 of 14) being positive, respectively. However, the genetic and epigenetic alterations of *p16*$^{INK4A}$ observed could not explain the lack of p16$^{INK4A}$ protein in 27 metastases, indicating the presence of additional inactivating mechanisms for p16$^{INK4A}$. Interestingly, *p16*$^{INK4A}$ promoter methylation was significantly overrepresented in *NRAS*-mutated samples compared to *NRAS* wild-type samples ($P=0.0004$), indicating an association between these two events.

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## INTRODUCTION

Cutaneous malignant melanoma (CMM) is a skin cancer with a poor prognosis at the metastatic stage (stage III–IV), and the disease shows a pronounced increase in incidence throughout Caucasian populations. The genetic and epigenetic aberrations known to be related to the development of CMM include inactivation of the *CDKN2A* gene by deletion, mutation, or promoter methylation (Serrano *et al.*, 1997; Ruas and Peters, 1998; Dahl and Guldberg, 2007). The *CDKN2A* gene has two alternative reading frames resulting in two unrelated tumor suppressor proteins, p16$^{INK4A}$ and p14$^{ARF}$, which are associated with the tumor suppressive functions of the RB protein and the p53 protein, respectively (Serrano *et al.*, 1997; Lowe and Sherr, 2003; Ha *et al.*, 2007). Mutations in *CDKN2A* are found both as somatic events in tumors, and rarely as germ-line alterations in the hereditary situation. Germ-line mutations, occur in approximately 25–40% of melanoma families (Hussussian *et al.*, 1994; Kamb *et al.*, 1994; Curtin *et al.*, 2005; Chin *et al.*, 2006). In Sweden, <10% of the familial melanoma kindreds are found to carry germ-line mutations of *CDKN2A* (Platz *et al.*, 1997). Somatic genetic aberrations in *CDKN2A* are dominated by deletions (30–60%) (Flores *et al.*, 1996; Funk *et al.*, 1998).

In addition, CMM etiology very commonly involves constitutive activation of the RAS–RAF–MEK–ERK signal transduction pathway, leading to enhanced proliferation and survival of tumor cells. In total, 70–90% of CMM metastases carry mutations in one of the proto-oncogenes *BRAF* or *NRAS* (Omholt *et al.*, 2003; Haluska *et al.*, 2006). However, prolonged oncogenic stimuli may also trigger cellular senescence through induction of p16$^{INK4A}$ and p53 proteins (Serrano *et al.*, 1997; Narita *et al.*, 2003; Michaloglou *et al.*, 2005). Thus, avoiding cell-cycle arrest and senescence during melanoma development possibly necessitates inactivation of *CDKN2A*, which influences both the p16$^{INK4A}$ and p53 pathways. This is an attractive explanation as to why *CDKN2A* is so frequently inactivated in melanoma.

Previously, we have screened for mutations in *NRAS* (exon 2) and *BRAF* (exons 11 and 15) in CMM metastases (Omholt *et al.*, 2003; Edlundh-Rose *et al.*, 2006). We have also previously characterized the prevalence of allelic losses of *CDKN2A* in these tumors (Grafstrom *et al.*, 2005). We found that samples with bi- or monoallelic deletions in the *INK4* region (containing the *CDKN2A* and *CDKN2B* genes, which code for the tumor suppressor proteins p16$^{INK4A}$, p14$^{ARF}$, and p15, respectively) had shorter median overall survival compared to patients without deletions. However, patients with tumors with biallelic deletions had the same overall survival as those with tumors with monoallelic deletions. This

[1]*Department of Oncology and Pathology, Cancer Center Karolinska, Karolinska Institutet/Karolinska University Hospital, Stockholm, Sweden*

[2]*These authors contributed equally to this work.*

*Correspondence: Suzanne Egyhazi, Department of Oncology and Pathology, Cancer Center Karolinska R8:03, S-171 76 Stockholm, Sweden. E-mail: suzanne.egyhazi@ki.se*

*Abbreviations: CMM, cutaneous malignant melanoma; IHC, immunohistochemistry; MSP, methylation-specific PCR; WT, wild-type*

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could be due to other mechanisms of gene inactivation in tumors that carry monoallelic deletions such as promoter methylation or mutations.

This study was aimed at investigating the prevalence of sporadic mutations and transcriptional silencing of *p16*$^{INK4A}$ by promoter methylation, in CMM metastases with monoallelic deletions and metastases without deletions in *p16*$^{INK4A}$. Furthermore, we wished to relate inactivation of *p16*$^{INK4A}$ to clinical and pathological parameters and the previously studied *NRAS* and *BRAF* oncogene mutation status in these metastases. To obtain a more complete picture of *p16*$^{INK4A}$ aberrations in melanoma metastases, we also included tumors with biallelic deletions.

## RESULTS

### *p16*$^{INK4A}$ promoter methylation

Promoter methylation of *p16*$^{INK4A}$ was observed in 15 of 59 (25%) metastases using methylation-specific PCR (MSP) (Figure 1, Table 1). Three of the tumors with biallelic deletions have been analyzed and none of them showed any promoter methylation, as expected because both alleles have been lost. Assuming that none of the tumors with biallelic deletions have any promoter methylation, *p16*$^{INK4A}$ promoter methylation occurred in 19% (15 of 77) of this cohort. Promoter methylation among tumors without *p16*$^{INK4}$ deletions was similar to those with monoallelic deletions, 7 of 21 (33%) and 8 of 35 (23%), respectively ($P=0.53$).

### *p16*$^{INK4A}$ mutation screening

Sequencing of *CDKN2A* exons 1α, 2, and 3 revealed that 9 of 56 (16%) metastases carried mutations in the coding region of *p16*$^{INK4A}$ (Table 1). Assuming that none of the tumors with biallelic deletions carry any mutation, 12% (9 of 77) of this cohort carried *CDKN2A* mutations. All *CDKN2A* mutations were heterozygous except in the M69 case that was hemi/homozygous. Interestingly, one metastasis (sample M12) had three different *p16*$^{INK4A}$ mutations (two nonsynonymous and one synonymous). A summary of all observed genetic variants found is shown in Supplementary Table S3 online. The two tandem mutations and four of the single-nucleotide substitutions showed classical UVB mutation signature patterns, i.e. G:C>A:T transitions at dipyrimidine sites, and GG:CC>AA:TT tandem alterations (Hocker and Tsao, 2007), indicating that several of the *p16*$^{INK4A}$ mutations were caused by UV exposure.

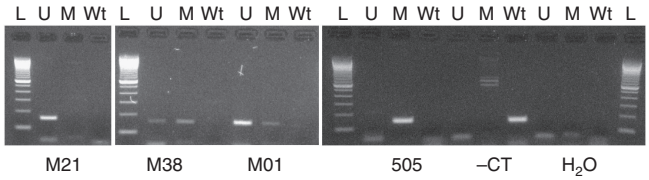


**Figure 1. Representative gel images from the methylation-specific PCR (MSP) analyses.** L = 100 bp DNA ladder, U/M/Wt = PCR products from the PCR specific for unmethylated, methylated, and non-CT converted-specific MSP reactions, respectively. Samples were M21, M38, and M01, and control reactions for the MSP were the methylation-positive cell line 505, Wt-positive CT-converted control DNA (-CT), and non-template control for PCR ($H_2O$). CT, bisulfite conversion of cytosine to thymidine.

Promoter methylation and *p16*$^{INK4A}$ mutation rarely coexisted—only sample M01 had both.

### Expression of the p16$^{INK4A}$ protein

A majority of the metastases lacked p16$^{INK4A}$ protein expression, including all those with *p16*$^{INK4A}$ promoter methylation (Table 1). In total, only 12 of 67 (18%) metastases were p16$^{INK4A}$ positive. As expected, none of the 11 analyzed tumors with biallelic deletions were p16$^{INK4A}$ positive. Assuming that all the tumors with biallelic deletions lack p16$^{INK4A}$ protein expression, 16% (12 of 77) of our cohort was p16$^{INK4A}$ positive. There were 7 of 21 (33%) p16-positive tumors without *p16*$^{INK4A}$ deletions, compared to 5 of 35 (14%) tumors with monoallelic deletions ($P=0.11$). In three cases only cytoplasmic staining was observed. Interestingly, three p16$^{INK4A}$-positive metastases (samples M12, M50, and M69) were also found to carry both a mutation in the coding region of *p16*$^{INK4A}$ and a monoallelic deletion. However, all samples with truncating mutations (i.e., p.E33X, p.Q50X, p.R80X, and p.W110X) and the tumor with a microdeletion causing a frameshift reading and truncated translation (p.A67fs145X) were negative for staining with the monoclonal p16$^{INK4A}$ antibody.

Furthermore, immunohistochemistry (IHC) analysis of the 14 primary tumors revealed that a majority of these expressed p16$^{INK4A}$ (10 of 14 positive, i.e. 71%) and that expression was lost in some of their corresponding metastases (6 of 14 positive, i.e. 43%) (Figure 2, Table 1). The primary tumors showed a more heterogeneous staining pattern for p16$^{INK4A}$ compared to the metastases, with negative areas (nests or clones) within the tumor. Unexpectedly, two cases negative for p16$^{INK4A}$ expression in the primary tumors were positive in the corresponding metastases (samples M24 and M66). An explanation could be that these primary tumors metastasized before they lost p16$^{INK4A}$ expression or that the metastases originated from minor p16$^{INK4A}$-positive clones within the primary tumors.

The number of p16$^{INK4A}$ positive tumors was significantly higher among tumors without any genetic or epigenetic alterations in comparison to tumors with one or two alterations, 7 of 11 (64%) and 5 of 56 (9%), respectively ($P=0.0002$). Noteworthy, 27 of 55 (49%) p16$^{INK4A}$ protein-expression-negative metastases had at least one intact *p16*$^{INK4A}$ allele (Table 1).

### Correlation of different p16$^{INK4A}$ aberrations with *NRAS* and *BRAF* oncogene mutation status

Interestingly, *p16*$^{INK4A}$ promoter methylation predominantly occurred in *NRAS*-mutated tumors with 12 of 15 (80%) methylated tumors carrying *NRAS* mutation (Table 1). Promoter methylation has been found in 12 of 23 (52%) *NRAS*-mutated metastases, 2 of 27 (7%) *BRAF*-mutated metastases, and 1 of 9 (11%) wild-type (WT) metastases (Table 2). The proportion of samples with *p16*$^{INK4A}$ promoter methylation among *NRAS*-mutated samples was significantly higher than the proportion in those with WT *NRAS* ($P=0.0004$).

This is in contrast to samples with *p16*$^{INK4A}$ mutations, which seemed to be more frequent in *BRAF*-mutated

**Table 1. Clinical and pathological characterization of the subjects in the study with data from the analyses of p16$^{INK4A}$**

| ID | Age at diagnosis (years) | Sex | Histopathological subtype | Breslow thickness (mm) | Oncogene mutated | p16$^{INK4A}$ deletion | p16$^{INK4A}$ promoter methylation | p16$^{INK4A}$/p14$^{ARF}$ mutation | p16$^{INK4A}$ protein metastasis (nuclear/cytoplasmic), primary tumor [nuclear/cytoplasmic] |
|---|---|---|---|---|---|---|---|---|---|
| M01 | 53 | M | SSM | 1.5 | BRAF | No | Methyl | **p.L63P** | (0/0) |
| M02 | 58 | F | SSM | 0.9 | BRAF | No | Unmethyl | **p.A67fs145X**/p.A123fs212X | (0/0) |
| M03 | 35 | F | SSM | 0.5 | BRAF | No | Unmethyl | **p.Q50X** | (0/0) |
| M04 | 51 | F | SSM | 6 | BRAF | No | Unmethyl | **p.R80X**/p.P135L | (0/0) |
| M05 | 42 | F | SSM | 2.2 | BRAF | No | Unmethyl | No | (0/0) |
| M06 | 70 | M | NM | 8 | BRAF | No | Unmethyl | No | (0/0) |
| M07 | 82 | M | SSM | 3.1 | BRAF | No | Unmethyl | No | (0/2) |
| M08 | 91 | M | NM | 13 | BRAF | No | Unmethyl | No | (0/3) |
| M09 | 79 | M | Unkn | NA | BRAF | No | Unmethyl | No | (2/3) |
| M10 | 71 | M | SSM | 0.5 | BRAF | Mono | Methyl | No | (0/0) |
| M11 | 46 | M | NM | 3 | BRAF | Mono | Unmethyl | **p.E33X** | (0/0) |
| M12 | 36 | M | SSM | 3.2 | BRAF | Mono | Unmethyl | **p.G23S**, **p.L97P** | (2/2) [3/3] |
| M13 | 69 | F | UC | 3.5 | BRAF | Mono | Unmethyl | No | (0/0) [0/0] |
| M14 | 62 | M | SSM | 1.6 | BRAF | Mono | Unmethyl | No | (0/0) [3/3] |
| M15 | 49 | M | Unkn | NA | BRAF | Mono | Unmethyl | No | (0/0) |
| M16 | 40 | M | SSM | 1.2 | BRAF | Mono | Unmethyl | No | (0/0) |
| M17 | 41 | F | SSM | 1.9 | BRAF | Mono | Unmethyl | No | (0/0) |
| M18 | 50 | M | Unkn | NA | BRAF | Mono | Unmethyl | No | (0/0) |
| M19 | 71 | M | UC | 7 | BRAF | Mono | Unmethyl | No | (0/0) |
| M20 | 68 | F | UC | 3 | BRAF | Mono | Unmethyl | No | (0/0) |
| M21 | 84 | F | SSM | 1.6 | BRAF | Mono | Unmethyl | No | (0/0) |
| M22 | 78 | F | UC | 0.7 | BRAF | Mono | Unmethyl | No | (0/0) |
| M23 | 34 | F | Unkn | NA | BRAF | Mono | Unmethyl | No | (0/0) |
| M24 | 45 | F | NM | 1.4 | BRAF | Mono | Unmethyl | No | (1/2) [0/0] |
| M25 | 57 | F | ALM | 0.8 | BRAF | Mono | Unmethyl | No | (1/2) |
| M26 | 40 | F | SSM | 4.8 | BRAF | Bi | Unmethyl | ND | (0/0) |
| M27 | 80 | M | UC | 3 | BRAF | Bi | Unmethyl | ND | (0/0) |
| M28 | 54 | M | SSM | 2.7 | BRAF | Bi | ND | ND | ND |
| M29 | 72 | F | SSM | 0.4 | BRAF | Bi | ND | ND | ND |
| M30 | 93 | M | NM | 7.1 | BRAF | Bi | ND | ND | ND |
| M31 | 24 | F | SSM | 0.3 | BRAF | Bi | ND | ND | ND |
| M32 | 78 | M | NM | 1.2 | BRAF | Bi | ND | ND | (0/0) |
| M33 | 59 | F | NM | 1.1 | BRAF | Bi | ND | ND | ND |
| M34 | 29 | M | SSM | 1 | BRAF | Bi | ND | ND | ND |
| M35 | 72 | M | SSM | 2.5 | NRAS | No | Methyl | No | (0/0) [0/1] |
| M36 | 58 | M | NM | 4.3 | NRAS | No | Methyl | No | (0/0) [0/2] |
| M37 | 76 | F | Unkn | NA | NRAS | No | Methyl | No | (0/0) |
| M38 | 65 | M | Unkn | NA | NRAS | No | Methyl | No | (0/0) |
| M39 | 72 | F | NM | 2.4 | NRAS | No | Methyl | No | (0/0) |
| M40 | 79 | M | UC | NA | NRAS | No | Methyl | No | (0/0) |

Table 1 continued on the following page

**Table 1. Continued**

| ID | Age at diagnosis (years) | Sex | Histopathological subtype | Breslow thickness (mm) | Oncogene mutated | p16$^{INK4A}$ deletion | p16$^{INK4A}$ promoter methylation | p16$^{INK4A}$/p14$^{ARF}$ mutation | p16$^{INK4A}$ protein metastasis (nuclear/cytoplasmic), primary tumor [nuclear/cytoplasmic] |
|---|---|---|---|---|---|---|---|---|---|
| M41 | 75 | F | NM | 1.4 | NRAS | No | Unmethyl | No | (0/0) |
| M42 | 77 | F | NM | 2.5 | NRAS | No | Unmethyl | No | (1/1) [3/3] |
| M43 | 24 | F | SSM | 1.4 | NRAS | No | Unmethyl | No | (1/1) |
| M44 | 75 | M | SSM | 5.3 | NRAS | Mono | Methyl | No | (0/0) [0/0] |
| M45 | 85 | M | NM | 9 | NRAS | Mono | Methyl | No | (0/0) [0/1] |
| M46 | 34 | F | NM | 3.8 | NRAS | Mono | Methyl | No | (0/0) [3/2] |
| M47 | 50 | M | NM | 3.5 | NRAS | Mono | Methyl | No | (0/0) |
| M48 | 79 | F | NM | 5.1 | NRAS | Mono | Methyl | No | (0/0) |
| M49 | 84 | F | NM | 2.7 | NRAS | Mono | Methyl | No | (0/0) |
| M50 | 53 | F | SSM | 1.1 | NRAS | Mono | Unmethyl | **p.P70L** | (2/3) [2/2] |
| M51 | 86 | F | UC | 0.9 | NRAS | Mono | Unmethyl | **p.W110X**/p.G166R | (0/0) |
| M52 | 69 | M | SSM | 1.6 | NRAS | Mono | Unmethyl | No | (0/0) [2/3] |
| M53 | 54 | F | UC | 0.8 | NRAS | Mono | Unmethyl | No | (0/0) |
| M54 | 71 | M | SSM | 1.3 | NRAS | Mono | Unmethyl | No | (0/0) |
| M55 | 69 | M | NM | 5 | NRAS | Mono | Unmethyl | No | (0/0) |
| M56 | 68 | M | SSM | 3 | NRAS | Mono | Unmethyl | No | (0/0) |
| M57 | 65 | M | Unkn | NA | NRAS | Mono | Unmethyl | No | (0/0) |
| M58 | 44 | M | SSM | 3.5 | NRAS | Bi | ND | ND | (0/0) |
| M59 | 81 | M | SSM | 4.4 | NRAS | Bi | ND | ND | ND |
| M60 | 45 | F | SSM | 1.8 | NRAS | Bi | ND | ND | ND |
| M61 | 53 | M | NM | 5 | NRAS | Bi | ND | ND | (0/0) |
| M62 | 73 | M | NM | 10.5 | NRAS | Bi | ND | ND | (0/0) |
| M63 | 50 | M | UC | 8 | NRAS | Bi | ND | ND | ND |
| M64 | 68 | F | UC | 1 | NRAS | Bi | ND | ND | ND |
| M65 | 47 | F | SSM | 0.7 | No | No | Unmethyl | No | (0/0) |
| M66 | 81 | M | SSM | 2.1 | No | No | Unmethyl | No | (1/1) [0/0] |
| M67 | 65 | M | ALM | 10 | No | No | Unmethyl | No | (1/2) [3/3] |
| M68 | 70 | F | SSM | 2.6 | No | Mono | Methyl | No | (0/0) |
| M69 | 58 | M | LMM | 0.5 | No | Mono | Unmethyl | **p.F90L**/p.L145P | (0/1) |
| M70 | 40 | F | NM | 7 | No | Mono | Unmethyl | No | (0/0) |
| M71 | 65 | M | NM | 10 | No | Mono | Unmethyl | No | (0/0) |
| M72 | 65 | F | UC | 3.5 | No | Mono | Unmethyl | No | (0/0) |
| M73 | 89 | M | SSM | 8.5 | No | Bi | Unmethyl | ND | (0/0) |
| M74 | 77 | F | ALM | 30 | No | Bi | ND | ND | (0/0) |
| M75 | 49 | F | SSM | 0.7 | No | Bi | ND | ND | (0/0) |
| M76 | 47 | M | NM | 2.6 | No | Bi | ND | ND | (0/0) |
| M77 | 68 | F | NM | 6 | No | Bi | ND | ND | (0/0) |

Abbreviations: ALM, acral lentigious melanoma; LMM, lentigo maligna melanoma; NA, not available; ND, not determined; NM, nodular melanoma; No, no mutation/deletion; SSM, superficial spreading melanoma; UC, unclassifiable; Unkn, patient with unknown primary tumor.
The boldface entries in the column for p16$^{INK4A}$/p14$^{ARF}$ mutation are related to P16$^{INK4A}$, and the non-bold entries next to the bold entries are related to P14$^{ARF}$.

metastases (6 of 25, 24%) compared to *NRAS*-mutated (2 of 23, 9%) and WT metastases (1 of 8, 12%) although this difference was not statistically significant ($P=0.27$) (Table 2).

However, the *NRAS* or *BRAF* mutation status shows little difference in the degree of inactivation of p16$^{INK4A}$ at the protein level (Table 2).

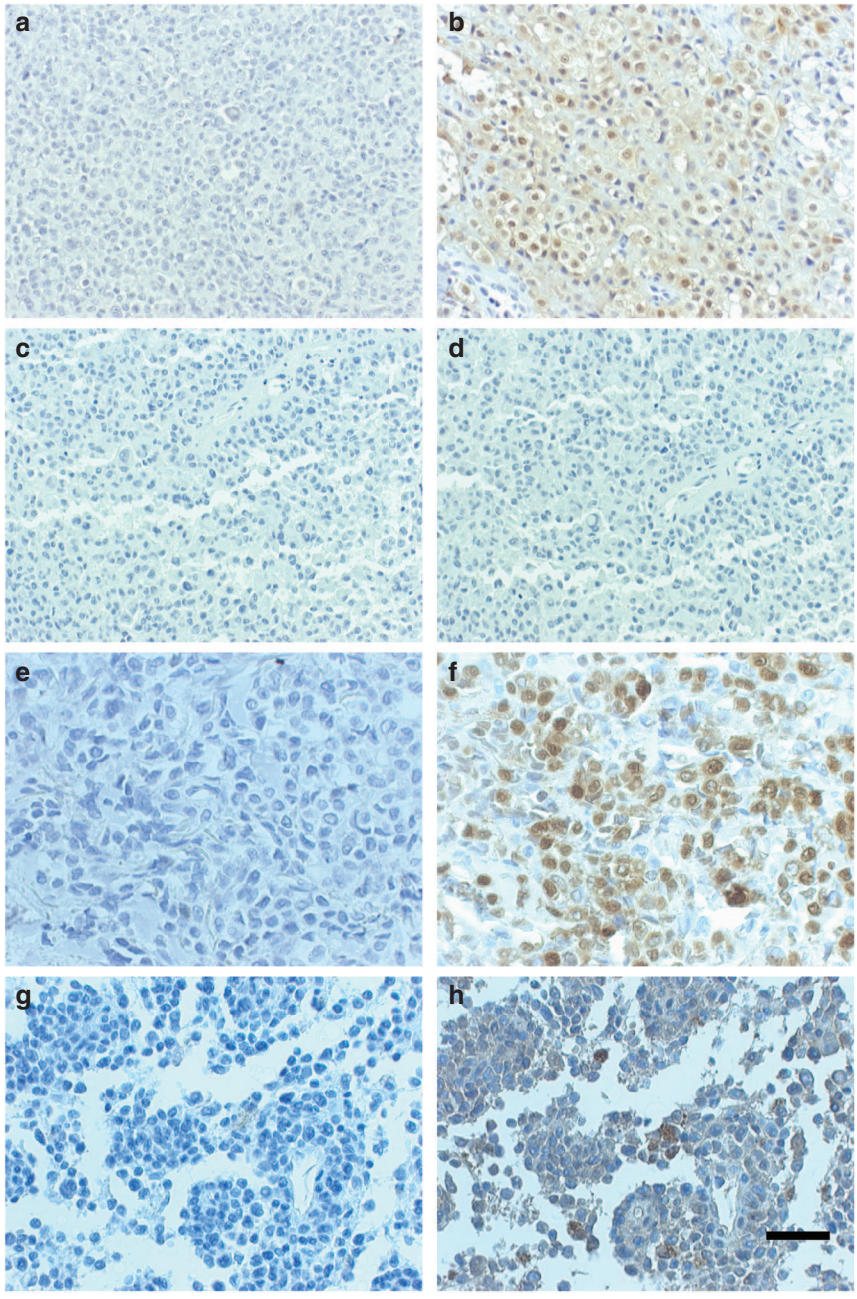


**Figure 2. Images of p16$^{INK4A}$ immunohistochemistry (IHC) sections.** Panels show the metastases M42 and M46 and their corresponding primary tumors: (**a**) primary M46, negative control; (**b**) primary M46, anti-p16$^{INK4A}$; (**c**) metastasis M46, negative control; (**d**) metastasis M46, anti-p16$^{INK4A}$; (**e**) primary M42, negative control; (**f**) primary M42, anti-p16$^{INK4A}$; (**g**) metastasis M42, negative control; (**h**) metastasis M42, anti-p16$^{INK4A}$. Both primary tumors and metastasis M42 were regarded positive for p16$^{INK4A}$ expression and M46 metastasis was regarded negative. Bar = 100 μm.

### Correlation of different p16$^{INK4A}$ aberrations with clinicopathological parameters

None of the clinicopathological parameters analyzed (gender, age at diagnosis, histopathological type, and Breslow thickness) showed any significant association with p16$^{INK4A}$ biallelic deletion, promoter methylation, mutation, or protein expression (data not shown). However, patients with tumors with *CDKN2A* mutations tended to have an earlier onset (median age, 53 years) than those lacking *CDKN2A* mutations (median age, 69 years) ($P = 0.053$).

## DISCUSSION

In this study, we analyzed *p16$^{INK4A}$* promoter methylation, mutation, and protein expression in tumors either lacking *p16$^{INK4A}$* deletions or having monoallelic or biallelic deletions. Although the smallest deletions are difficult to detect and to correctly assign the tumors in their respective deletion categories, we consider that the data presented in this work is accurate enough to give a good picture of the total impact of *p16$^{INK4A}$* aberrations in melanoma metastases. The major obstacles of performing high-quality analyzes on excised

**Table 2. Summary of the alterations of *p16*$^{INK4A}$ in relation to *NRAS* and *BRAF* oncogene mutation status**

| | Total (*n*=77) | *BRAF* (*n*=34) | *NRAS* (*n*=30) | WT (*n*=13) |
|---|---|---|---|---|
| *p16*$^{INK4A}$ *deletion* | | | | |
| No | 21 (27%) | 9 (26.5%) | 9 (30%) | 3 (23%) |
| Mono | 35 (45%) | 16 (47%) | 14 (47%) | 5 (38.5%) |
| Bi | 21 (27%) | 9 (26.5%) | 7 (23%) | 5 (38.5%) |
| *p16*$^{INK4A}$ *promoter methylation** | | | | |
| No | 44 (75%) | 25 (93%) | 11 (48%) | 8 (89%) |
| Yes | 15 (25%) | 2 (7%) | 12 (52%) | 1 (11%) |
| ND | 18 | 7 | 7 | 4 |
| *p16*$^{INK4A}$/*p14*$^{ARF}$ *mutation* | | | | |
| No | 47 (84%) | 19 (76%) | 21 (91%) | 7 (88%) |
| Yes | 9 (16%) | 6 (24%) | 2 (9%) | 1 (12%) |
| ND | 21 | 9 | 7 | 5 |
| *p16*$^{INK4A}$ *protein expression* | | | | |
| No | 55 (82%) | 22 (79%) | 23 (88%) | 10 (77%) |
| Yes | 12 (18%) | 6 (21%) | 3 (12%) | 3 (23%) |
| ND | 12 | 6 | 6 | 0 |

Abbreviation: ND, not determined.
**P*=0.0004.

tumor material are the presence of nontumor cells in the samples, degradation of DNA in archival tumor tissues and during the bisulfite treatment, and the cellular heterogeneity within the tumors. For these reasons, the included tumors and sections were selected so as to have high percentages of tumor cells (>70%), the PCR reactions were designed to be compatible with moderately degraded DNA, and evaluation of IHC sections was performed for multiple regions.

We found that *p16*$^{INK4A}$ promoter methylation occurred in 19% of the metastases (including the tumors with biallelic deletions), which is similar to previous studies, reporting between 5 and 19% in primary melanomas and 27 and 33% in metastases (Straume *et al.*, 2002; Sharpless and Chin, 2003; Freedberg *et al.*, 2008). There was a somewhat higher proportion of methylated samples among tumors without *p16*$^{INK4A}$ deletions (33% methylated) compared to those with monoallelic deletions (23% methylated), but this difference was not statistically significant. Our results indicate that monoallelic deletions and promoter methylation are not mutually excluding events, and both alterations contribute to p16$^{INK4A}$ inactivation. However, all samples with promoter methylation were negative for p16$^{INK4A}$ protein, suggesting that both alleles are silenced, although this has to be confirmed using allele-specific methylation analyses.

An interesting finding was that 52% (12 of 23) of the *NRAS*-mutated tumors had *p16*$^{INK4A}$ promoter methylation compared to only 7% (2 of 27) of the *BRAF*-mutated tumors. The preferential coexistence of *p16*$^{INK4A}$ promoter methylation and *NRAS* mutation in the same tumors may be related to a cooperation of these genetic changes. Despite that tumors with *NRAS* or *BRAF* mutations show little difference in the degree of inactivation of p16$^{INK4A}$ at the protein level (88 vs. 79% inactivation, respectively) and both oncogene mutations are known to induce senescence in melanocytes (Narita *et al.*, 2003; Michaloglou *et al.*, 2005), the sequence of events leading to neoplastic development may be different. The senescence inducing effect of *NRAS* mutation may be more pronounced and faster, thereby necessitating rapid (or preexisting) inactivation of *p16*$^{INK4A}$ whereas *BRAF* mutations might coexist with a functional p16$^{INK4A}$ protein early in the tumor development.

Another possible explanation for the differential existence of promoter methylation and *NRAS* mutation compared to methylation and *BRAF* mutation could be presence of a mechanistic link between establishment of the *NRAS* mutation and the events leading to aberrant promoter methylation of *p16*$^{INK4A}$. Support for such a link is that the transcription factor E2F, downstream of ras, has been shown to influence DNA methyltransferase 1 activity in a mouse model (Kimura *et al.*, 2003). In addition, H-ras transformation has been shown to be accompanied by increased levels of DNA methyltransferase activity and repression of several genes by methylation. Treatment of 5-aza-deoxycytidine or suppression of oncogenic ras led to reexpression of these genes showing that oncogenic ras activity is linked to epigenetic silencing (Lund *et al.*, 2006).

Overall, 12% (including the tumors with biallelic deletions) of our samples carried *p16*$^{INK4A}$ mutations, which is similar to previous reports (around 8%) on cutaneous melanoma (Hocker and Tsao, 2007; Freedberg *et al.*, 2008). In malignant melanoma, the majority of somatic mutations in *CDKN2A* have been shown to exhibit UVR signature (Hocker and Tsao, 2007). Several of the p16$^{INK4A}$ mutations observed in this study were C>T or CC>TT transitions, typically caused by UVB exposure.

In several studies, it has been found that p16$^{INK4A}$ protein expression is gradually lost during melanoma progression: almost all benign nevi are positive for p16$^{INK4A}$ (74–100%), whereas primary melanomas show reduced levels of p16$^{INK4A}$ expression (28–78% p16 positive) and, in metastases, only 6–24% of samples are p16$^{INK4A}$ positive (Ghiorzo *et al.*, 2004a; Mihic-Probst *et al.*, 2006; Fearfield *et al.*, 2007; Sanki *et al.*, 2007). Our results are in line with other reports on p16$^{INK4A}$ expression. In our study 16% of the metastases (including tumors with biallelic deletions) expressed p16$^{INK4A}$ protein. Three of these had both a monoallelic deletion and carried a mutation in the coding region. We and others have found that p16$^{INK4A}$ mutation-positive tumors express the protein although it has been shown that some of these point mutations impair the function of p16$^{INK4A}$ protein (Ghiorzo *et al.*, 2004b; Scaini *et al.*, 2009). In addition (Yang *et al.*, 2005) predicted the effects of 117 reported *CDKN2A* point mutations showing large variations regarding the impact on p16$^{INK4A}$ protein.

However, lack of expression of $p16^{INK4A}$ protein in 49% of the samples with at least one functional gene copy could not be fully explained by the genetic and epigenetic alterations identified in our study. The reasons for this could be misclassification of tumors with biallelic deletions into (primarily) the monoallelic deletion group in particular if tumors with deletions spanning only few kilobases are common. This is unlikely because pattern of deletion mapping suggest that small deletions are uncommon (Gonzalgo *et al.*, 1997; Palmieri *et al.*, 2000).

Other means of inactivation might also take place in these samples—for example, repressors of $p16^{INK4A}$ and other epigenetic events apart from promoter methylation. One such mechanism might be transcriptional repression by β-catenin (Delmas *et al.*, 2007). Constitutive activation of the Wnt/β-catenin pathway is common in melanoma, and Delmas *et al.* (2007) reported that activated β-catenin in cooperation with activated N-ras promotes melanoma development in mice. Posttranscriptional micro-RNA-mediated suppression of $p16^{INK4A}$ translation by miR-24 has also been reported (Lal *et al.*, 2008).

In summary, we have characterized mechanisms and patterns of $p16^{INK4A}$ inactivation in human melanoma. In our study $p16^{INK4A}$ is most commonly inactivated by deletions and/or promoter methylation (Table 2). Although the analysis of *CDKN2A* is limited by the lack of study of $p14^{ARF}$, we found that four of nine mutations in exon 2 also affect the $p14^{ARF}$ protein (Table 1). In addition, we have previously shown that $p14^{ARF}$ is lost in 67% of the cases with $p16^{INK4A}$ biallelic deletions and that $p14^{ARF}$ biallelic deletions rarely occur without simultaneous loss of $p16^{INK4A}$ (Grafstrom *et al.*, 2005). We found interesting patterns regarding inactivation of $p16^{INK4A}$ in melanoma metastases, in relation to *NRAS* and *BRAF* oncogene mutation status—possibly reflecting genetic and epigenetic alterations that occur during melanoma development and progression. Intriguingly, $p16^{INK4A}$ promoter methylation predominantly occurred in *NRAS*-mutant cases indicating an association between the two events.

## MATERIALS AND METHODS

### Patients and tumor samples

A total of 77 melanoma metastases, including 21 with biallelic deletions, from equal number of melanoma patients who underwent surgery between 1992 and 2002 at the Karolinska University Hospital were included in the study. Both formalin-fixed, paraffin-embedded archival samples and fresh-frozen tissue samples were used for DNA extraction. In addition, 14 corresponding primary tumors were available for analysis of $p16^{INK4A}$ protein expression. Approval has been obtained from the ethics committee of Karolinska Institutet. Patients have given informed consent. The study has been conducted according to the Declaration of Helsinki Principles. Patient characteristics with clinical and pathological parameters are summarized in Supplementary Table S1 online and given in Table 1 for the individual cases.

The relative allelic concentrations of *p16INK4A* in intron 1 (immediately 5′ of exon 2) had been analyzed previously by quantitative real-time RT-PCR (Grafstrom *et al.*, 2005).

### DNA extraction

DNA was extracted from two to four 10-μm-thick slices of formalin-fixed, paraffin-embedded tissue using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA was also extracted from frozen tissue samples in 10 cases, using the same kit.

### Methylation-specific PCR

After bisulfite treatment of DNA, promoter methylation of $p16^{INK4A}$ was analyzed by MSP, using primers specific for unmethylated (U) and methylated (M) sequences. Bisulfite conversion of DNA was performed using the EZ DNA methylation Gold Kit (Zymo Research, Orange, CA) following the manufacturer's instructions. DNA (500 ng) was used for each bisulfite conversion reaction, which was performed at least in duplicate for each sample. The completeness of the bisulfite conversion reaction was controlled using non-CT converted specific PCR for all samples (WT primers) (Herman *et al.*, 1996). Primer sequences are given in Supplementary Table S2 online.

Approximately 100 ng bisulfite-modified DNA was used in each (U, M, and WT) PCR, which also contained primers (2 μM per primer); dNTPs (each 400 μM), 1 × PCR buffer, 1.5 mM $MgCl_2$; 2 U of Platinum Taq polymerase (Invitrogen, Paisley, UK); and 1% BSA (New England Biolabs, Hitchin, UK), in a reaction volume of 20 μl. DMSO (5%; Sigma-Aldrich Sweden AB, Stockholm, Sweden), was used in the WT mix. PCR conditions were 3 minutes at 95 °C, 36 cycles × (20 seconds at 94 °C, 15 seconds at annealing temperature, 20 seconds at 72 °C), then 5 minutes at 72 °C, and soak at 10 °C.

In addition, a nested PCR approach was used for samples with low initial DNA concentration. Bisulfite-converted DNA was then amplified in a two-step PCR procedure using previously published outer primers (Holst *et al.*, 2003). The first PCR conditions were as described above except reduced number of cycles (20 cycles) (30 cycles in the MSP).

PCR products were separated on 1.6% agarose gels, stained with ethidium bromide, and visualized under UV light. A sample was regarded positive for methylation when at least two different PCRs from separate bisulfite conversion reactions yielded M products. DNA from cell line A375 was used as an unmethylated control and DNA from cell line 505 was used as a methylated control. Cell line DNA not modified with sodium bisulfite was used as WT control, and water replaced DNA in the contamination control reaction.

### Sequencing of $p16^{INK4A}$

Exons 1α, 2, and 3 of *CDKN2A* ($p16^{INK4A}$) were PCR amplified (primer sequences and annealing temperatures are found in Supplementary Table S2 online). DNA extracted from frozen tissue was amplified using primers spanning the whole exon 2, whereas paraffin-extracted DNA was amplified in two overlapping fragments. Approximately 100 ng DNA was used in each PCR as follows: 3 minutes at 95 °C, 40 cycles × (20 seconds at 94 °C, 20 seconds at annealing temperature, 20 seconds at 72 °C), then 5 minutes at 72 °C, and soak at 12 °C. Samples that yielded low amounts of PCR products were amplified further in a nested PCR. PCR products (2 μl) from the first reaction were then amplified for additional 24 cycles. All PCR products were enzymatically treated with shrimp alkaline phosphatase and exonuclease I (50 minutes at 37 °C, followed by

20 minutes at 80 °C). Bidirectional sequencing was performed using the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and same primers as for PCR. Products were sequenced on an automated sequencer (ABI3130XL; Applied Biosystems). To confirm mutations, we reamplified samples and sequenced them again.

### Immunohistochemistry

IHC was performed on 4 μm, formalin-fixed, paraffin-embedded sections. Heat-induced antigen retrieval was performed in Reveal solution in a decloaking chamber (Biocare, Concord, CA) according to the manufacturer's instructions, and thereafter rinsed in hot rinse. Briefly, sections were incubated overnight (4 °C) with the monoclonal primary p16$^{INK4A}$ antibody, clone JC8 (Biocare) diluted 1:250 in Tris-buffered saline buffer with 1.5% horse serum. Negative controls were incubated without primary antibody. Secondary antibody incubation using streptavidin/peroxidase complex was according to a kit manual (Vectastain Universal Quick Kit; Vector Laboratories, Burlingame, CA) as was development with 3,3′-diaminobenzidine (Immunkemi, Stockholm, Sweden). Finally, sections were counterstained with Mayer's hematoxylin. Normal breast tissue and a melanoma tumor positive for p16$^{INK4A}$ were included in all IHC batches.

Independent evaluation of all slides was first performed by three observers (AJ, SE, and EG), thereafter a consensus was reached regarding the p16$^{INK4A}$ protein expression. The intensity was scored (0 = absent, 1 = weak, 2 = moderate, 3 = strong staining) for nuclear and cytoplasmic staining. Samples scored 1 or higher were considered positive. Samples with $<5\%$ positive cells were regarded negative (score = 0).

### Statistical methods

Statistical tests were two-sided and *P*-values exceeding 95% confidence level were considered significant (*t*-test, $\chi^2$-test, or Fisher's exact test when appropriate).

## CONFLICT OF INTEREST

The authors state no conflict of interest.

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## SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

## REFERENCES

Chin L, Garraway LA, Fisher DE (2006) Malignant melanoma: genetics and therapeutics in the genomic era. *Genes Dev* 20:2149–82

Curtin JA, Fridlyand J, Kageshita T *et al.* (2005) Distinct sets of genetic alterations in melanoma. *N Engl J Med* 353:2135–47

Dahl C, Guldberg P (2007) The genome and epigenome of malignant melanoma. *Apmis* 115:1161–76

Delmas V, Beermann F, Martinozzi S *et al.* (2007) Beta-catenin induces immortalization of melanocytes by suppressing p16INK4a expression and cooperates with N-Ras in melanoma development. *Genes Dev* 21:2923–35

Edlundh-Rose E, Egyhazi S, Omholt K *et al.* (2006) NRAS and BRAF mutations in melanoma tumours in relation to clinical characteristics: a study based on mutation screening by pyrosequencing. *Melanoma Res* 16:471–8

Fearfield LA, Larkin JM, Rowe A *et al.* (2007) Expression of p16, CD95, CD95L and Helix pomatia agglutinin in relapsing and nonrelapsing very thin melanoma. *Br J Dermatol* 156:440–7

Flores JF, Walker GJ, Glendening JM *et al.* (1996) Loss of the p16INK4a and p15INK4b genes, as well as neighboring 9p21 markers, in sporadic melanoma. *Cancer Res* 56:5023–32

Freedberg DE, Rigas SH, Russak J *et al.* (2008) Frequent p16-independent inactivation of p14ARF in human melanoma. *J Natl Cancer Inst* 100:784–95

Funk JO, Schiller PI, Barrett MT *et al.* (1998) p16INK4a expression is frequently decreased and associated with 9p21 loss of heterozygosity in sporadic melanoma. *J Cutan Pathol* 25:291–6

Ghiorzo P, Mantelli M, Gargiulo S *et al.* (2004a) Inverse correlation between p16INK4A expression and NF-kappaB activation in melanoma progression. *Hum Pathol* 35:1029–37

Ghiorzo P, Villaggio B, Sementa AR *et al.* (2004b) Expression and localization of mutant p16 proteins in melanocytic lesions from familial melanoma patients. *Hum Pathol* 35:25–33

Gonzalgo ML, Bender CM, You EH *et al.* (1997) Low frequency of p16/CDKN2A methylation in sporadic melanoma: comparative approaches for methylation analysis of primary tumors. *Cancer Res* 57:5336–47

Grafstrom E, Egyhazi S, Ringborg U *et al.* (2005) Biallelic deletions in INK4 in cutaneous melanoma are common and associated with decreased survival. *Clin Cancer Res* 11:2991–7

Ha L, Ichikawa T, Anver M *et al.* (2007) ARF functions as a melanoma tumor suppressor by inducing p53-independent senescence. *Proc Natl Acad Sci USA* 104:10968–73

Haluska FG, Tsao H, Wu H *et al.* (2006) Genetic alterations in signaling pathways in melanoma. *Clin Cancer Res* 12:2301s–7s

Herman JG, Graff JR, Myohanen S *et al.* (1996) Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 93:9821–6

Hocker T, Tsao H (2007) Ultraviolet radiation and melanoma: a systematic review and analysis of reported sequence variants. *Hum Mutat* 28:578–88

Holst CR, Nuovo GJ, Esteller M *et al.* (2003) Methylation of p16(INK4a) promoters occurs *in vivo* in histologically normal human mammary epithelia. *Cancer Res* 63:1596–601

Hussussian CJ, Struewing JP, Goldstein AM *et al.* (1994) Germline p16 mutations in familial melanoma. *Nat Genet* 8:15–21

Kamb A, Gruis NA, Weaver-Feldhaus J *et al.* (1994) A cell cycle regulator potentially involved in genesis of many tumor types. *Science* 264:436–40

Kimura H, Nakamura T, Ogawa T *et al.* (2003) Transcription of mouse DNA methyltransferase 1 (Dnmt1) is regulated by both E2F-Rb-HDAC-dependent and -independent pathways. *Nucleic Acids Res* 31:3101–13

Lal A, Kim HH, Abdelmohsen K *et al.* (2008) p16(INK4a) translation suppressed by miR-24. *PLoS ONE* 3:e1864

Lowe SW, Sherr CJ (2003) Tumor suppression by Ink4a-Arf: progress and puzzles. *Curr Opin Genet Dev* 13:77–83

Lund P, Weisshaupt K, Mikeska T *et al.* (2006) Oncogenic HRAS suppresses clusterin expression through promoter hypermethylation. *Oncogene* 25:4890–903

Michaloglou C, Vredeveld LC, Soengas MS *et al.* (2005) BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature* 436:720–4

Mihic-Probst D, Mnich CD, Oberholzer PA *et al.* (2006) p16 expression in primary malignant melanoma is associated with prognosis and lymph node status. *Int J Cancer* 118:2262–8

Narita M, Nunez S, Heard E *et al.* (2003) Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* 113:703–16

Omholt K, Platz A, Kanter L *et al.* (2003) NRAS and BRAF mutations arise early during melanoma pathogenesis and are preserved throughout tumor progression. *Clin Cancer Res* 9:6483–8

Palmieri G, Cossu A, Ascierto PA *et al.* (2000) Definition of the role of chromosome 9p21 in sporadic melanoma through genetic analysis of primary tumours and their metastases. The Melanoma Cooperative Group. *Br J Cancer* 83:1707–14

Platz A, Hansson J, Mansson-Brahme E *et al.* (1997) Screening of germline mutations in the CDKN2A and CDKN2B genes in Swedish families with hereditary cutaneous melanoma. *J Natl Cancer Inst* 89:697–702

Ruas M, Peters G (1998) The p16INK4a/CDKN2A tumor suppressor and its relatives. *Biochim Biophys Acta* 1378:F115–77

Sanki A, Li W, Colman M *et al.* (2007) Reduced expression of p16 and p27 is correlated with tumour progression in cutaneous melanoma. *Pathology* 39:551–7

Scaini MC, Rossi E, de Siqueira Torres PL *et al.* (2009) Functional impairment of p16(INK4A) due to CDKN2A p.Gly23Asp missense mutation. *Mutat Res* 671:26–32

Serrano M, Lin AW, McCurrach ME *et al.* (1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* 88:593–602

Sharpless E, Chin L (2003) The INK4a/ARF locus and melanoma. *Oncogene* 22:3092–8

Straume O, Smeds J, Kumar R *et al.* (2002) Significant impact of promoter hypermethylation and the 540 C>T polymorphism of CDKN2A in cutaneous melanoma of the vertical growth phase. *Am J Pathol* 161:229–37

Yang G, Rajadurai A, Tsao H (2005) Recurrent patterns of dual RB and p53 pathway inactivation in melanoma. *J Invest Dermatol* 125:1242–51